AWARD NUMBER:
W81XWH-14-1-0400

TITLE:
Modular Bioconjugates to Study Herceptin Resistance: A Structural and Functional Approach

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	15Sep2014 - 14Sep2015

4. TITLE AND SUBTITLE
Modular Bioconjugates to Study Herceptin Resistance: A Structural and Functional Approach

5a. CONTRACT NUMBER
W81XWH-14-1-0400

5b. GRANT NUMBER
BC134050

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Berkeley
Berkeley, CA 94704-5940

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
During the course of these studies, the method for attachment of antibodies to the exterior surface of bacteriophage MS2 capsids was optimized, and the conjugates were fully characterized by a series of biophysical methods. Furthermore, and most importantly, these conjugates were shown to retain their affinity and specificity for their epitopes, and staining procedures for application to mass cytometry experiments were developed, and a proof of concept experiment has been completed. The results of these studies were recently published.

15. SUBJECT TERMS
Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	24	19b. TELEPHONE NUMBER *(include area code)*

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1. Introduction

Understanding the mechanisms by which breast cancers develop resistance to therapy is an important and active area of research. Breakthroughs in this area have the potential to identify unforeseen targets for therapy, with the potential to even develop synthetic lethalities through targeting multiple pathways at once. Resistance to Herceptin therapy represents a particularly troubling case due to the high number of breast cancers that overexpress the HER2 receptor. The overall goal of the present work is directed toward understanding structural and functional changes that take place within breast cancer cells that leads to the observed resistance phenotype. In order to study this system, a collection of modular bioconjugates will be synthesized that will allow in depth profiling of the relevant pathways. In this reporting period, the major focus has been on synthesizing, characterizing, and validating conjugates targeted to the HER2 and EGFR receptors.

2. Keywords

bioconjugation, oxidative coupling, viral capsids, antibodies, HER2, EGFR, mass cytometry, flow cytometry

3. Accomplishments

Major Goals of Project:

Below are the stated tasks in the approved statement of work. Text in bold+italics provides information on the progress relative to the stated goals and/or milestones.

Task 1. Preparation and validation of targeted, modular mAb-MS2 bioconjugates (months 1-36 as needed throughout the duration of the project):

1a. Refinement of protocol for attachment of mAb to MS2 capsids (months 1-6, ***completed***).

1b. Evaluation of the specificity of these conjugates by flow/mass cytometry, confocal microscopy, and electron microscopy (months 6-12 and as needed as new targets are explored and need validation, ***characterization and validation completed for HER2 and EGFR which were used for proof of concept***).

*Milestone(s) Achieved: Preparation and validation of the conjugates to be used for the ensuing studies. Publication of these results in a peer-reviewed journal (12 months, **results published and attached in Appendix**).*

Task 2. The study of morphological changes with respect to invadopodia formation in cells upon treatment with Herceptin (months 12-30):

1a. TEM studies of healthy cells and tumorigenic cells with conjugates described in Task 1 (months 6-18, ***0% completed***).

1b. iLEM studies of tumorigenic cells that have been cultured in 2D and 3D in response to prolonged Herceptin exposure (months 18-30, ***0% completed***).

*Milestone(s) Achieved: A full assessment of the role, if any, that invadopodia structures play in Herceptin resistance mechanisms (30 months, **0% completed**). Publication of results in peer-reviewed journals (36 months).*

Task 3. The study of signaling dynamics in HER2-overexpressing breast cancer cells in response to various clinically relevant therapeutics (months 12-36):
1a. Development of a signaling fingerprint of >10 breast cancer cell lines using cyTOF before treatment with Herceptin and other clinically relevant therapies (months 6-18, ***0% completed***).
1b. CyTOF studies of the signaling dynamics that change upon initial and prolonged treatment of the aforementioned cell lines with clinically relevant therapies (months 18-30, ***0% completed***).
1c. In-depth data analysis to uncover patterns arising in the results (months 6-36, ***0% completed***).
*Milestone(s) Achieved: Preparation of cyTOF agents for detection of ultra-low abundance analytes (24 months). Network analysis of the signaling dynamics in breast cancer cells in response to Herceptin treatment (30 months, **0% completed**). Publication of results in peer-reviewed journals (36 months).*

Accomplishments Relative to Goals:

For this period of reporting, experimental effort was almost exclusively spent optimizing the protocol for preparing the agents that will be necessary for subsequent studies (i.e. Task 1 above). Furthermore, these conjugates were fully characterized and demonstrated to maintain selective and efficient binding to their required epitopes in model studies that included fluorescence-based flow cytometry, confocal microscopy, and mass cytometry experiments. The results of this work were published in mid-June 2015, and the manuscript that details these efforts is included in Appendix A and is summarized below.

A key objective of the proposed research requires the preparation and validation of antibody-MS2 bioconjugates through a modular strategy that allows for the rapid and facile preparation of a panel of agents that are capable of being detected in a variety of modes. To this end, we developed a two-stage approach to their preparation. Namely, genome-free MS2 viral capsids that bear a non-natural cysteine residue at position 87 (i.e. N87C mutant) on the interior surface of the capsid and an unnatural para-amino phenylalanine (paF) at position 19 (i.e. T19paF) were expressed in *E. coli* and are readily purified by sequential anion exchange chromatography, ammonium sulfate precipitation, and size exclusion chromatography. These capsids are first functionalized on the interior surface by taking advantage of cysteine-maleimide reactivity. For this particular work, fluorophores or metal chelators were attached to the interior surface using protocols previously developed in the Francis group.

In order to append the antibodies to the exterior surface of the capsids, the antibodies were chemically modified by treatment with 5 equivalents of an NHS ester/nitrophenol heterobifunctional cross-linking reagent. Using 5 equivalents of this cross-linking reagent proved optimal as it ensured that >98% of antibodies were modified at least once with the reagent, as determined from integrations of reconstructed high resolution mass spectral data obtained independently on the heavy and light chains (see Figures 1a,b on page 1591 of Appendix A) . Upon completion of the reaction, the solution is treated with sodium dithionite ($Na_2S_2O_4$) to chemoselectively reduce the nitrophenol moiety to the corresponding aminophenol oxidative coupling precursor. These antibodies are then added to a buffered solution of MS2 capsids in a 3:1 ratio of

antibody to capsids and treated with sodium periodate ($NaIO_4$) to promote the oxidative coupling reaction, which we found is complete in 2 minutes (see Figures 1c,d on page 1591 of Appendix A). Here, size exclusion chromatography of the reaction demonstrated that upon reaction completion, all of the antibody ends up attached to the MS2 viral capsids. When a higher number of equivalents of antibodies is used, however, some antibody remains free in solution, making extensive purification of the conjugates necessary. By utilizing a 3:1 ratio, minimal purification is necessary since all of the antibody ends up attached to the viral capsids. As expected, a size difference of the conjugate relative to the unmodified capsids is observed by both TEM and DLS measurements. Importantly, the TEM measurements demonstrate that the capsid structure remains intact (see Figure 2 on page 1592 of Appendix A).

With characterization of the conjugates completed, we required validation that these agents maintain the ability to bind to their antigens. As such, MS2 viral capsids that had been modified with an Oregon Green fluorescent dye on their interior surface were prepared, and the antibody conjugation was performed with the optimized protocol. Flow cytometry was then performed using the HCC1954 cell line (HER2 and EGFR positive), and efficient binding to these cells was noted when an anti-EGFR antibody was attached to the exterior surface of the MS2 capsid (Figure 3a on page 1593 of Appendix A). Furthermore, a K_d was determined for our construct that indicated that these constructs maintain their binding affinity upon attachment to the capsid (Figure 3b,c on page 1593 of Appendix A). Furthermore, live-cell confocal microscopy clearly demonstrated binding to cells in their native, adhered state (Figure 4 on page 1594 of Appendix A). Somewhat surprisingly, however, we found that these capsids are rapidly internalized relative to their fluorescently-labeled antibody counterparts. Although the mechanism by which this process occurs is beyond the scope of the current work, the facile uptake of the targeted capsids suggests that these conjugates may find additional value as drug delivery vehicles.

As a final proof of concept for our preliminary studies, we developed a staining protocol to label cells with our agents that were modified with reporters for mass cytometry in line with Specific Aim 3. Initial experiments (data not shown) indicated a high degree of background nonspecific interaction of the capsids with cells when using the traditional washing protocols for mass cytometry (i.e. water washes after fixation and labeling). The use of phosphate buffers is generally problematic in mass cytometry as the high salt content can lead to highly diminished signal upon prolonged exposure to the buffer. After much optimization, we found that washing the cells 3 times with low salt, 10 mM phosphate buffer and performing a final resuspension in water allowed for low background signal, and minimal instrument down-time was required for cleaning (see Figure 5 on page 1594 of Appendix A).

Training/Professional Development:

During the training period, a number of training and professional development opportunities were undertaken. Training opportunities were completed through training 2 graduate students in the laboratory (Chawita Netirojjanakul and Ioana Aanei) during the course of the research toward the major accomplishments described above as well as through training a first year graduate rotation student (Emily Hartman) in our laboratory. Professional development opportunities pursued can be subdivided into two

major activities: coursework and presentations at conferences/invited seminars and are described in detail below. In addition, a review article was written with Prof. Francis regarding the oxidative couplings developed in our laboratory that have made possible the preparation of the agents described in the work related to this proposal.

Coursework:

- Completion of MCB132 (Molecular Biology of Human Cancer) at UC Berkeley. This course covered a diverse array of topics, including tumor pathology, intracellular signaling, tumor supressors, oncogenes, multi-step carcinogenesis, invasion, metastasis, genetic instability, and epidemiology.
- Completion of an introductory course in immunology through iTunes-U (i.e. self-study) that covered topics encompassing the adaptive and innate immune systems, including antibody structure, function, and production and the various elements of the complement system and the interactions of the two systems with each other.

Presentations:

- A. M. ElSohly. "New Materials Enabled by Rapid and Efficient Oxidative Coupling Reactions." *An invited seminar at the weekly Dept. of Chemistry seminar series at the University of Mississippi*, September 2015
- I. L. Aanei, A. M. ElSohly, C. Netirojjanakul, M. E. Farkas, J. W. Gray, and M. B. Francis. "Targeted Viral Capsids as Imaging Agents for Breast Cancer Detection." *A poster presented at the Breast Oncology Program Scientific Retreat*, January 2015.
- A. M. ElSohly. "New Protein-Based Materials Empowered by Oxidative Coupling Reactions." *A talk delivered during the Molecular Foundry Seminar Series at the Lawrence Berkeley National Laboratory*, November 2014

Dissemination to Communities of Interest:
Nothing to report

Plans for Next Reporting Period:

With agents validated, in the next reporting period, I plan to begin work in utilizing the described agents to study the signaling networks and invadopodia formation as described in Tasks 2 and 3 above.

4. Impact

Principal Discipline:
The novel nanoscale scaffolds developed in the work during this reporting period provides a modular method to rapidly prepare a series of conjugates targeted to specific receptors that are overexpressed on the surface of many breast cancer lesions. By segregating the reporter species from the targeting group by using a viral capsid protein shell, secondary effects from the reporter species are highly mitigated.

Other Disciplines:
None to report.

Technology Transfer:
Nothing to report.

Greater Society:
Nothing to report.

5. Changes/Problems

Changes in Approach:
Nothing to report

Problems/Delays and Resolutions:
The majority of the reported work in the Appendix went quite smoothly; however, the mass cytometry staining protocol required extensive and unforeseen optimization. In traditional mass cytometry experiments, sample fixation and washing in pure water are performed prior to sample analysis. With our agents, however, extensive background signal in negative control samples was observed when samples were washed with only water. After extensive optimization, we found that samples washed with 10 mM buffer displayed greatly reduced background and led to specific detection of the receptors of interest. While these problems did not delay our efforts toward Task 1, the effort that was required to solve this issue precluded work toward either of the other tasks.

Changes Impacting Expenditures:

Although current expenditures have been far under budget, it is anticipated that as more of the work for Tasks 2 and 3 above are performed, our expenditures will increase substantially to the point of remaining on-budget.

6. Products

Journal Publications:

- A. M. ElSohly, C. Netirojjanakul, I. L. Aanei, A. Jager, S. C. Bendall, M. E. Farkas, G. P. Nolan, and M. B. Francis. Synthetically Modified Viral Capsids as Versatile Carriers for Use in Antibody-based Cell Targeting. *Bioconjugate Chem.* **2015**, *26*, 1590–1596. Published with acknowledgement of federal support and attached as Appendix A.
- A. M. ElSohly and M. B. Francis. Development of Oxidative Coupling Strategies for Site-Selective Protein Modification. *Acc. Chem. Res.* **2015**, *48*, 1971–1978. Published with acknowledgement of federal support and attached as Appendix B.

7. Participants & Other Collaborating Organizations

Individuals:

Name:	Adel ElSohly
Project Role:	Postdoc (PI)
Person Months Worked:	12
Contribution to Project:	Conducted all aspects of research, including design and execution of experiments
Funding Support	This grant (BC134050)

Name:	Chawita Netirojjanakul
Project Role:	Graduate Student
Person Months Worked:	5
Contribution to Project:	Assisted in optimization of labeling protocols and determining binding affinities/specificities
Funding Support	HHMI international graduate student fellowship

Name:	Ioana Aanei
Project Role:	Graduate Student
Person Months Worked:	5
Contribution to Project:	Assisted in optimization of mass cytometry labeling protocol and TEM characterization of conjugates
Funding Support	Genentech graduate fellowship

Changes in Active Other Support:
Nothing to report.

Other Organizations:

Organization Name:	Stanford University
Location:	Paolo Alto, CA (USA)
Organization Contribution:	Facilities

8. Special Reporting Requirements

Nothing to report

9. Appendices

Appendix A: Reprint of “Synthetically Modified Viral Capsids as Versatile Carriers for Use in Antibody-based Cell Targeting.”

Appendix B: Reprint of “Development of Oxidative Coupling Strategies for Site-Selective Protein Modification.”

Bioconjugate Chemistry

Article

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Synthetically Modified Viral Capsids as Versatile Carriers for Use in Antibody-Based Cell Targeting

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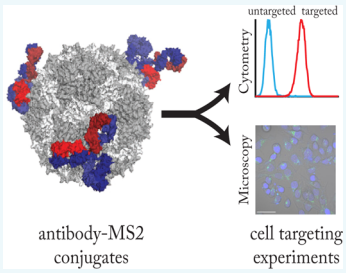
Supporting Information

ABSTRACT: The present study describes an efficient and reliable method for the preparation of MS2 viral capsids that are synthetically modified with antibodies using a rapid oxidative coupling strategy. The overall protocol delivers conjugates in high yields and recoveries, requires a minimal excess of antibody to achieve modification of more than 95% of capsids, and can be completed in a short period of time. Antibody−capsid conjugates targeting extracellular receptors on human breast cancer cell lines were prepared and characterized. Notably, conjugation to the capsid did not significantly perturb the binding of the antibodies, as indicated by binding affinities similar to those obtained for the parent antibodies. An array of conjugates was synthesized with various reporters on the interior surface of the capsids to be used in cell studies, including fluorescence-based flow cytometry, confocal microscopy, and mass cytometry. The results of these studies lay the foundation for further exploration of these constructs in the context of clinically relevant applications, including drug delivery and in vivo diagnostics.



INTRODUCTION

Nanoscale carriers, such as polymers,[1,2] dendrimers,[3,4] inorganic nanoparticles,[5,6] and liposomes,[7,8] have been useful in many applications, including fundamental research, drug delivery, and diagnostic imaging. In addition to these synthetic scaffolds, self-assembled multimeric biomolecular complexes, such as heat shock proteins[9−11] and viral capsids,[12−17] have also shown great promise for the development of next generation imaging and drug delivery agents. The interior cavities and multiple attachment sites of these protein cage scaffolds allow them to house a large amount of imaging or therapeutic payloads, leading to enhancement of the signal intensity and the ability to deliver multiple copies of drug molecules. However, in order to achieve specific detection or delivery, these vehicles must be modified with targeting agents. Correspondingly, studies have increasingly demonstrated the importance of active targeting in achieving appropriate intratumoral localization.[18] Various chemical bioconjugation techniques have played crucial roles in the development of these targeted protein cage nanoparticles using different types of targeting groups, including small molecules,[19,20] nucleic acid aptamers,[15] peptides,[10,21,22] glycans,[23] or antibodies.[10,24] Among the different types of targeting agents, antibodies have been most widely used for a variety of applications due to their general availability as well as high specificity and affinity to targets. Numerous antibodies have been used as research tools or developed into diagnostic or imaging agents; furthermore, a growing number of antibodies (more than 20 to date) are being approved as therapeutic agents targeting specific ligands or receptors.[25−27]

Despite their excellent targeting ability, antibodies have a limited capacity for cargo delivery. Only a small number of modifications can be made on the surface of the antibody without either losing binding to the desired target or reducing efficacy through increased clearance.[28] In addition, drug molecules can induce precipitation of the antibody at high levels of modification due to their hydrophobicity. Great efforts have been dedicated to the optimization of antibody−drug conjugates (ADC), with several now in clinical trials or even available as treatments.[29] The use of viral capsids as delivery vehicles offers a number of advantages to traditional ADC systems. These protein assembles can carry over 100 copies of a given drug molecule, offering significant increases in therapeutic index and allowing the use of less cytotoxic agents. Furthermore, many drugs that are unsuitable for high levels of conjugation to antibodies due to hydrophobicity could be appended inside the capsid without precipitation of the conjugate. Finally, conjugation of drug molecules would not impede epitope binding by virtue of the drug cargo being located inside the capsid.

Received: April 22, 2015
Revised: June 12, 2015
Published: June 15, 2015

DOI: 10.1021/acs.bioconjchem.5b00226
Bioconjugate Chem. 2015, 26, 1590−1596

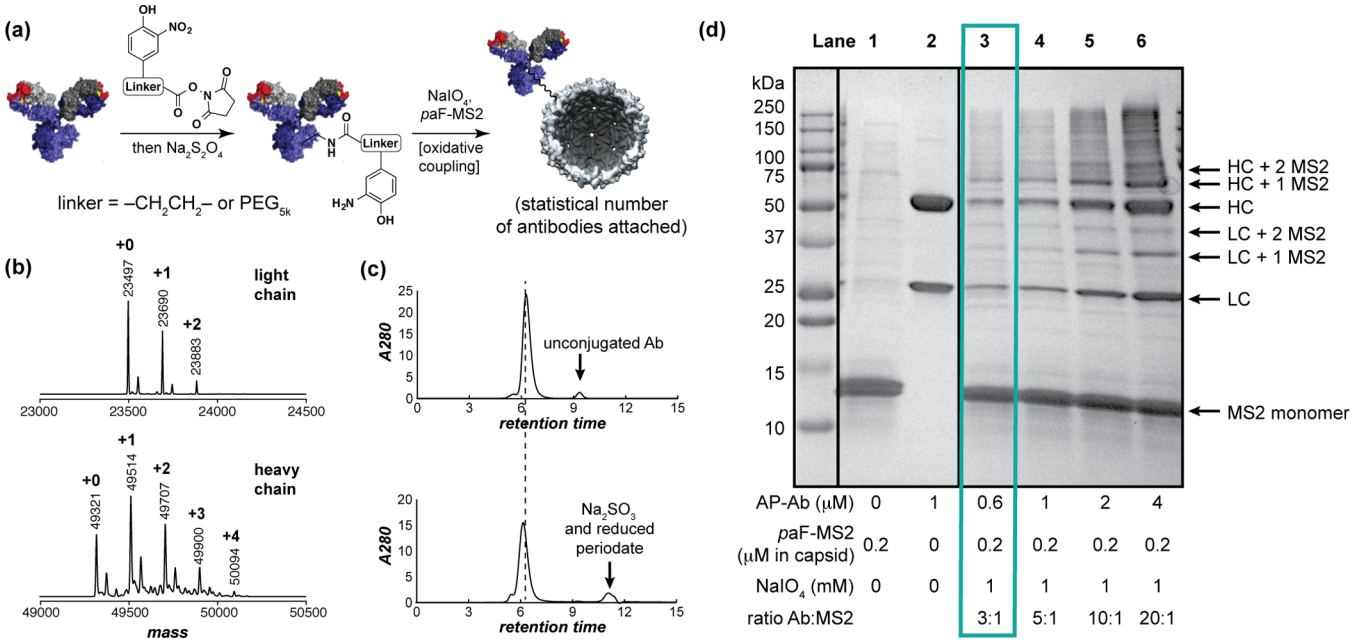


Figure 1. Generation of MS2–antibody conjugates. (a) General synthetic scheme of MS2–antibody conjugates. First, nitrophenol (NP) groups were attached to antibodies via lysine modification using a NP-NHS ester. The nitrophenol groups were then reduced to yield aminophenol-Ab conjugates (AP-Ab) by addition of $Na_2S_2O_4$. The resulting AP-Ab was coupled to *p*aF MS2 via oxidative coupling using $NaIO_4$. (b) LC-MS analysis of humanized anti-HER2 antibodies after lysine modification with 5 equiv of NP-NHS. The light chains (LC) were either unmodified (59%) or modified with one (34%) or two (6%) NP groups. The heavy chains (HC) were modified with 0 (16%), 1 (40%), 2 (31%), 3 (12%), or 4 (1%) NP groups. (c) SEC-HPLC of the oxidative coupling of humanized AP-anti-EGFR and *p*aF-MS2 when a 3:1 ratio of antibody:capsid was used indicates complete consumption of the antibody. The top trace was taken before the addition of sodium periodate, and the bottom trace was taken after addition of sodium sulfite to quench the reaction after 6 min. The peak at ~11.5 min in the latter trace is due to sodium sulfite. (d) SDS-PAGE analysis of MS2-anti-EGFR conjugates using 3, 5, 10, and 20 equiv of Ab with respect to the MS2 capsid concentration. The gel showed conjugation of one or two MS2 monomers to the light and heavy chains of antibodies. More equivalents of Ab resulted in a higher intensity of the modified bands.

Two previous reports have delineated methods for preparing antibody–viral capsid and antibody-heat shock protein conjugates. Both relied on the use of a heterobifunctional maleimide/*N*-hydroxy succinimide (NHS) ester linker,[10,24] and these constructs were successful at specifically targeting and killing cells expressing the receptor of interest when loaded with cytotoxic payloads. These reports did not indicate the effect that conjugation has on the binding affinity of the antibody. Additionally, the synthetic strategies required a large amount of antibody (i.e., high concentration) and extended reaction times.

In this work, we describe the preparation and characterization of a panel of MS2-antibody (MS2-Ab) conjugates using a facile and modular approach that is rapid, results in stoichiometric attachment, and exhibits little interchain cross-linking. Furthermore, the activation of the antibody component prior to coupling yields a stable species that can be stored for subsequent use, a feature that is not possible with maleimides or NHS esters. Biophysical and biological assessments of the MS2-Ab conjugates indicate comparable binding affinity relative to the parent antibodies. Finally, we demonstrate the use of MS2-Ab constructs to detect cell surface receptors via flow cytometry, confocal microscopy, and mass-cytometry.[30,31] The potential of signal enhancement provided by the MS2 scaffold and the high binding specificity and affinity of antibodies can be expanded toward many other applications, including imaging and drug delivery. Moreover, we anticipate that the method presented here can be readily adapted for the generation of a wide range of targeted nanoscale carriers.

RESULTS AND DISCUSSION

Antibody Modification and Attachment to MS2 Viral Capsids. Previous work in our laboratory has demonstrated the utility of genome-free bacteriophage MS2 viral capsids as delivery vehicles for imaging agents[17,32] and therapeutic drugs.[16,33] The capacity of MS2 to load up to 180 copies of small molecules inside the 27 nm icosahedral capsid make it particularly useful for enhancing the intensity of imaging agents or delivering multiple copies of drugs in one carrier. To direct these carriers to specific targets, we have employed several classes of targeting agents, including cyclic peptides,[21] linear peptides,[34] and DNA aptamers.[15] However, monoclonal antibodies (mAbs) provide a large collection of targeting agents that would provide much value to these carriers.

Due to the large sizes of both the antibody (Ab) and MS2 viral capsid, we chose a highly efficient oxidative coupling of aminophenols and anilines to conjugate the two entities. This reaction has been shown to couple two large biomolecules under mild conditions with very short reaction times.[15,21,35,36] Aniline-containing MS2 viral capsids were obtained via unnatural amino acid incorporation of *p*-aminophenylalanine (*p*aF) on the exterior surface.[37,38] The aminophenol moiety was introduced onto the antibodies via non-site-specific lysine modification using a nitrophenol-NHS ester (NP-NHS). When ready for attachment to MS2, the nitro group was reduced chemoselectively to the corresponding aminophenol with sodium dithionite. $NaIO_4$ was then used as the oxidant to couple the two partners (Figure 1a).

DOI: 10.1021/acs.bioconjchem.5b00226
Bioconjugate Chem. 2015, 26, 1590–1596

First, to investigate the possibility of generating MS2-Ab conjugates, a humanized anti-HER2 mAb was used as a model substrate. A screen to find the optimal number of nitrophenol groups to be attached to the mAb revealed that using 5 equiv of NP-NHS was optimal and resulted in ∼40% of the light chains and ∼84% of the heavy chains having at least one NP (Figure 1b and SI Figures 1 and 2) by LC-MS. These levels of modification corresponded to a product distribution wherein 99% of the full-sized mAbs have at least one NP attached (see the Supporting Information for full details). The use of large excesses (>20 equiv) of NP-NHS resulted in extensive interchain cross-linking in the subsequent oxidative coupling step (see SI Figure 3) and did not appreciably improve the coupling conversion. As such, 5 equiv of NP-NHS were used for the generation of AP-mAb in all subsequent experiments. After reducing the nitrophenols to aminophenol groups, these AP-mAb were subjected to trial oxidative coupling reactions with *p*aF-MS2 in 3:1 and 5:1 antibody to capsid ratios, both of which demonstrated clear conjugation to the desired constructs. Importantly, this coupling reaction could be performed on very small scales (10 μL reactions) under high dilution (as low as 200 nM antibody concentration) in less than 10 min (typically under 5 min). Furthermore, this coupling was efficient with humanized (Figure 1) and mouse-derived (SI Figure 3) antibodies, suggesting that differences in the constant regions of antibodies are compatible with this protocol.

To optimize the ratio of mAb per capsid, we varied the number of anti-EGFR mAb equivalents (3, 5, 10, and 20) in the oxidative coupling reaction with *p*aF MS2. The conjugation was confirmed by SDS-PAGE analysis (Figure 1d). Importantly, as more equivalents of antibody were used relative to capsid, an increase in the intensity of bands corresponding to conjugated product was observed, suggesting that increased numbers of antibodies are attached to the capsids. Here, we note that the presence of unmodified light and heavy chain bands is not due to unconjugated antibodies, as any excess was easily removed during purification (vide infra); rather, these bands are due to the chains of the antibody that are not directly attached to the capsid.

To determine the amount of unconjugated Ab remaining after the reactions, we monitored the extent of coupling when 3, 5, 10, and 20 equiv of AP-anti-EGFR are used by high performance liquid chromatography on a size exclusion column (see SEC HPLC traces in SI Figure 4). Reaction mixtures were analyzed prior to the addition of oxidant (precoupling) and following quenching of the periodate with Na_2SO_3 (post-coupling). The reaction of MS2 with 3 equiv of anti-EGFR resulted in no free mAb (Figure 1c), while the reaction with 5 equiv showed only a trace of uncoupled mAb. Increased amounts of free mAb remained when 10 or 20 equiv were added; however, the excess unconjugated mAb could be removed by performing successive spin concentration with molecular weight cutoff (MWCO) of 100 kDa, as confirmed by SEC HPLC. We also found that the MS2-anti-EGFR conjugates derived from a higher number of anti-EGFR equivalents elute at shorter retention times, suggesting an increase in the size of MS2-mAb conjugates relative to the unmodified capsid (SI Figure 5). While these results indicated that the number of equivalents of antibody bound to the capsid can be varied, we favored the 3:1 mAb:MS2 ratio since statistically >95% of the conjugates would be expected to have at least one mAb appended per viral capsid (see Supporting Information and SI Figure 6 for full details). These conditions also require minimal purification as all the antibodies are consumed in the coupling with the MS2 capsid.

Biophysical Characterization of MS2-Ab Conjugates. Given the size change indicated by a shorter SEC HPLC retention time, we pursued a more in-depth size characterization of these constructs. Dynamic light scattering (DLS) measurements of conjugates prepared with an initial 3:1 ratio of mAb:MS2 indicated a size of 30.73 ± 0.80 nm (Figure 2a), only

(a)

Sample	Measured size (nm)
MS2	27.07 ± 1.16
MS2-αEGFR (3 eq)	30.73 ± 0.80
MS2-αEGFR (5 eq)	30.07 ± 0.78
MS2-αEGFR (10 eq)	33.34 ± 1.85
MS2-αEGFR (20 eq)	40.11 ± 0.41

(b)

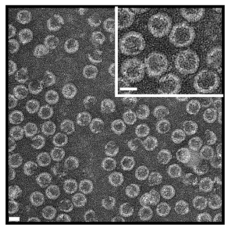


Figure 2. Biophysical characterization of MS2-anti-EGFR conjugates. (a) Size of MS2-anti-EGFR conjugates using varying numbers of equivalents of aminophenol-containing anti-EGFR antibodies as starting materials. Diameters were calculated from an average of three measurements by dynamic light scattering (DLS), shown as size distribution by number, which weights large and small particles equally. (b) Transmission electron micrograph (TEM) images of MS2-Ab conjugates using 3 equiv of AP-anti-EGFR. The capsids were shown to be intact, and their measured diameter was 31 nm. The scale bar represents 20 nm.

∼3 nm larger than the unmodified capsid, which measured 27.07 ± 1.16 nm. This small size difference suggested that the antibodies attached such that the C2 axis of the antibody is tangential, rather than perpendicular, to the surface of the MS2 capsid. Increasing the number of mAb attached to MS2 resulted in an increase in the hydrodynamic radius of the conjugates (Figure 2a). Transmission electron microscopy (TEM) allowed a direct size measurement of 31 nm, consistent with the data obtained from DLS. Importantly, the images showed intact capsid after antibody conjugation and purification (Figure 2b).

Binding Studies of MS2-Ab Conjugates. To test the utility of the MS2-mAb constructs, we analyzed whether the specificity and affinity of the Ab were retained after conjugation to the MS2 capsid. First, Oregon Green 488 dyes (OG) were attached to the mutated Cys (N87C) residue on the interior of MS2 via thiol-maleimide chemistry (SI Figure 7). MS2-OG capsids were then conjugated to a humanized IgG1 mAb that targets epidermal growth factor receptor (EGFR) overexpressed on the cell surface of many cancer types. Using flow cytometry, we analyzed the binding specificity and affinity of the MS2-anti-EGFR conjugates in an EGFR-negative (MCF7 clone 18) and three EGFR-positive (MDA-MB-231, L3.6pl, and HCC1954) human-derived cancer cell lines. The cells were incubated for 45 min on ice with 8 nM MS2 capsid (1.5 μM monomer concentration) in binding buffer (DPBS + 1% FBS). The MS2-anti-EGFR constructs only bound to the EGFR-positive cells, while no binding was observed with the EGFR negative cells (Figure 3a). The untargeted MS2-OG and MS2-OG conjugated to a nonspecific human IgG1 were used as negative controls, neither of which exhibited binding to any of the cell lines tested. This result thus confirmed that the binding of MS2-anti-EGFR conjugates was specifically due to the EGFR/anti-EGFR mAb interaction. Additional experiments using MS2-anti-HER2 on HER2-positive cells are provided in

DOI: 10.1021/acs.bioconjchem.5b00226
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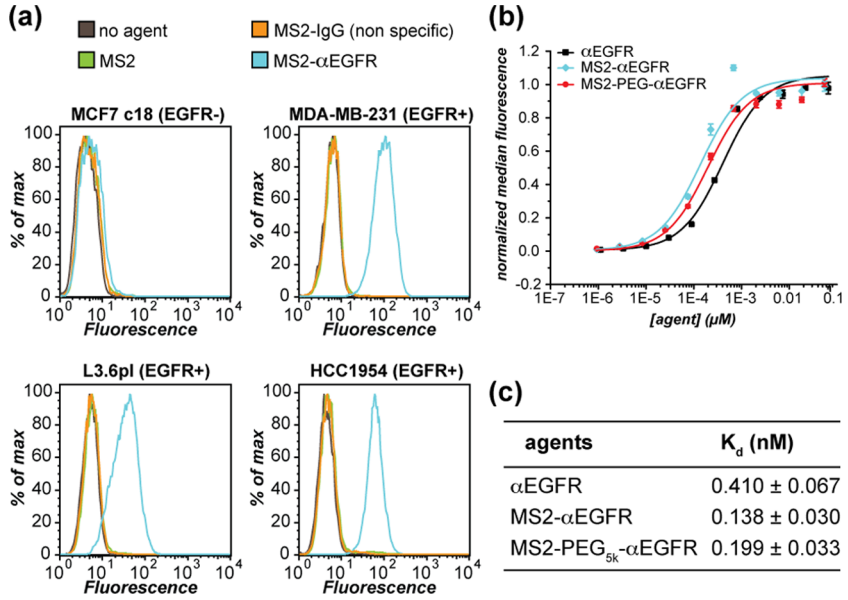

Figure 3. Binding studies of MS2-anti-EGFR antibody conjugates. (a) Flow cytometry analysis of the binding of Oregon Green 488 (OG)-containing MS2-anti-EGFR Ab conjugates to EGFR negative (MCF7 clone 18) and EGFR positive (MDA-MB-231, L3.6pl, and HCC1954) cell lines. The results from flow cytometry showed specific binding of MS2-anti-EGFR conjugates to only the EGFR positive cell lines, while remaining unbound to the EGFR negative cells. MS2-OG and MS2-OG conjugated to nonspecific human IgG1 were used as negative control agents. (b) Comparison of binding affinities of unmodified anti-EGFR antibodies, MS2-anti-EGFR, and MS2-PEG_{5k}-anti-EGFR conjugates. Flow cytometry was used to measure the median fluorescence of MDA-MB-231 cell populations after incubation with the samples. The data were fitted to a single-site binding model. K_d values ± the standard error of each fit are listed in (c).

SI Figure 8. In addition, we prepared MS2-mAb conjugates with a 5 kDa poly(ethylene glycol) (PEG) spacer to investigate whether further improvements to the binding of the MS2-mAb conjugates could be achieved by allowing the antibody to orient more favorably during the binding events. First, nitrophenol-capped 5k-PEG-acid was conjugated to anti-EGFR via lysine modification (see Figure 1a). The nitrophenol groups were reduced to aminophenols (AP), and the AP-PEG-anti-EGFR conjugates were attached to *p*aF MS2 using the same oxidative coupling strategy described above.

Next, we compared the binding affinity of unmodified anti-EGFR antibodies and MS2-anti-EGFR conjugates using MDA-MB-231 breast cancer cells. By fitting the median fluorescence intensity measurement from each point to a single site binding model, we determined the K_d of unmodified anti-EGFR and MS2-anti-EGFR conjugates to be equal to 0.41 ± 0.07 nM and 0.14 ± 0.03 nM, respectively (Figure 3b,c). The K_d of the anti-EGFR mAb was in excellent agreement with another published study of ^{125}I cetuximab binding to EGFR on MDA-MB-231, which reported a K_d of 0.38 nM.[39] Therefore, the conjugation to MS2 was not deleterious to the binding affinity. The binding affinity of MS2-PEG_{5k}-anti-EGFR was assessed by flow cytometry, and the K_d was calculated to be 0.20 ± 0.03 nM, suggesting that the PEG spacer did not decrease the binding affinity substantially relative to MS2-anti-EGFR, and furthermore, targets with sterically demanding epitopes may benefit from such a construct.

Live-Cell Confocal Imaging Studies. Next, we elected to probe the interaction of MS2-mAb conjugates with live cells as a function of time. HCC1954 and MCF7-clone 18 cells were treated with OG-labeled MS2-anti-EGFR and MS2-anti-HER2, respectively, at a concentration of 5.5 nM conjugate for 1 h at 37 °C in DPBS with 1% FBS binding buffer. After incubation, excess conjugates were washed away, and the cells were placed in dye-free media and imaged immediately to provide a 0 h baseline (Figure 4a). The cells were then placed back in an incubator at 37 °C for 1.5 h, and a second round of images were acquired (Figure 4b). After a total of 6 h at 37 °C post-labeling, DAPI was added, and the final images were taken (Figure 4c). Immediately after washing, some signal was already observed in the interior of the cells for both cell lines. As the cells were allowed to interact with surface-bound agent for longer time periods, increased amounts of signal was observed in what appears to be vesicle-like formations, and by 6 h, no discernible signal could be detected on the cell surface. EGFR is known to undergo internalization upon binding to a series of anti-EGFR antibodies, including cetuximab.[40−44] In fact, receptor downregulation and internalization is one of the main mechanisms of action of anti-EGFR therapeutic antibodies.[39] A control experiment utilizing fluorescently labeled anti-EGFR antibodies on HCC1954 cells showed strong labeling of the cell surface (SI Figure 9) at early time points. Intriguingly, the MS2-mAb conjugates were internalized at a higher rate, with most of the signal from agents present within the cells as opposed to on the cell membrane. Taken together, these data suggest that MS2-mAb conjugates targeted to highly overexpressed cell surface receptors may facilitate internalization, possibly through engagement of multiple cell surface receptors by multiple antibodies on the surface of a single capsid. Importantly, control experiments with nontargeted capsids (i.e., either without antibodies or with a nontargeted IgG control on the surface) were not found to bind these cells (SI Figure 10).

Application of MS2-Ab Conjugates in Mass Cytometry. Mass cytometry has emerged as a powerful tool in studying cell signaling events through receptor/protein profiling. This technology uses lanthanide metal isotopes for detection by inductively coupled plasma time-of-flight (ICP-TOF) mass spectrometry, thus allowing as many as 100

DOI: 10.1021/acs.bioconjchem.5b00226
Bioconjugate Chem. 2015, 26, 1590−1596

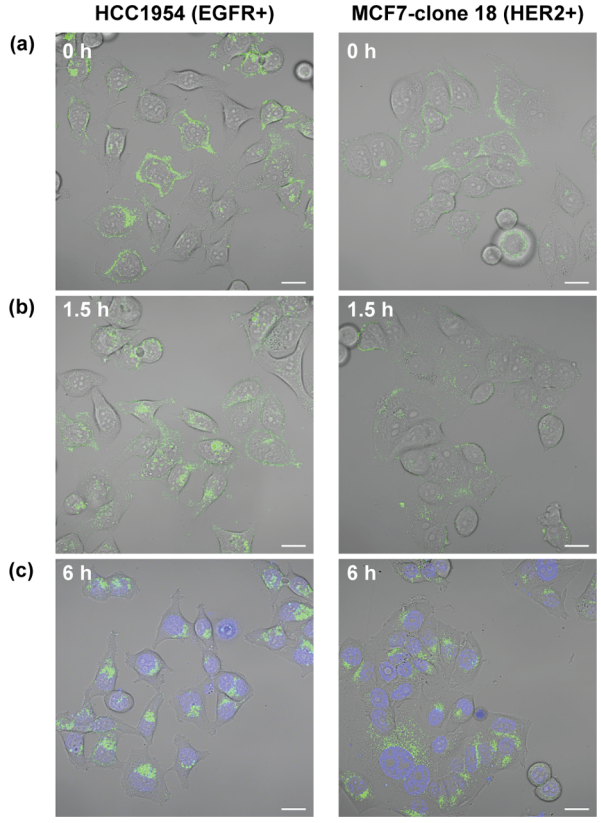

Figure 4. Live-cell confocal microscopy studies of MS2-Ab binding to breast cancer cells. (a) HCC1954 (EGFR+) and MCF7-clone 18 (HER2+) cells were incubated with fluorescently labeled MS2-anti-EGFR and MS2-anti-HER2, respectively, at a concentration of 5.5 nM capsid for 1 h at 37 °C. The cells were washed, placed in fresh dye-free media, and imaged immediately. After imaging, the cells were incubated at 37 °C in the absence of further agent for 1.5 h and were subsequently imaged (b). After a total of 6 h post-incubation at 37 °C, DAPI was added, and the final images were taken (c). Rapid internalization is observed in both cell lines, with the majority of the signal localized inside the cell even 1.5 h after removal of the unbound agent. The scale bars represent 50 μm.

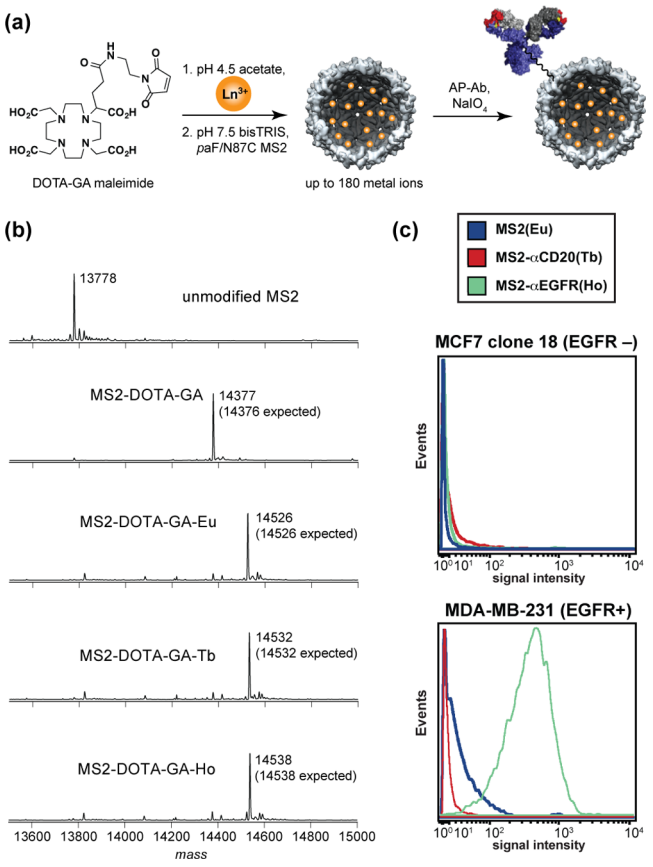

Figure 5. Mass cytometry analysis of binding interactions of lanthanide-chelated agents with cells. (a) Synthetic scheme summarizing synthesis of lanthanide-containing MS2-Ab conjugates. In situ chelation and conjugation of the lanthanide with DOTA-GA maleimide and subsequent oxidative coupling delivers the desired conjugate. (b) LC-MS characterization of the MS2-lanthanide constructs demonstrating that near quantitative conversion occurs. All spectra are reconstructed, and the values represent $[M + H]^+$ ions. (c) Mass cytometry using MCF-7 clone 18 (EGFR and CD20 negative) and MDA-MB-231 (EGFR positive, CD20 negative) costained with a panel of agents. Graphs are plotted as channel overlays of histograms with an arcsinh of 45 applied to all data sets. High levels of staining were only observed for agents displaying EGFR antibodies with EGFR positive cells. Additional controls and cell lines are included in SI Figure 11.

parameters to be measured simultaneously.[45] This number represents a dramatic increase over traditional fluorescence-based flow cytometry, which is limited to ~15 channels due to spectral overlap. In addition, when using lanthanide ions for detection, there is zero background signal from endogenous sources, which provides high sensitivity over a large dynamic range. Here, we took advantage of the MS2 capsid as a vehicle capable of carrying a large number of metal ions for specific detection of proteins of interest in mass cytometry.

As a proof of concept, we generated a series of MS2-lanthanide mass cytometry staining reagents, including MS2-(Ho)-anti-EGFR, MS2(Ho)-PEG_{5k}-anti-EGFR, MS2(Eu) (untargeted control), and MS2(Tb)-anti-CD20 (negative control). These constructs were prepared by initial chelation of the lanthanide ion of interest with DOTA-GA-maleimide followed by in situ conjugation to the interior cysteines of the MS2 capsid (Figure 5a). The use of the DOTA-GA chelator proved optimal (as compared to DOTA-maleimide) in the development of this one-pot procedure due to the increased solubility of the lanthanide-chelated species in aqueous solutions (Figure 5b). Subsequent conjugation of the antibodies provided the desired panel of agents. For these studies, we used HCC1954 and MDA-MB-231 cell lines, which are known to overexpress the EGFR and not the CD20 receptor. The MCF7 clone 18 cell line was used as the negative control. Cells were stained with a mixture of 3 agents that included either MS2(Ho)-anti-EGFR or MS2(Ho)-PEG_{5k}-anti-EGFR as the targeted reporter in addition to MS2(Eu) and MS2(Tb)-anti-CD20 as controls. The simultaneous treatment with multiple agents dramatically reduces the number of samples to be examined and limits the variability among them.

Consistent with our fluorescence-based flow cytometry results, we observed specific binding of the targeted agents to the cells expressing EGFR (Figure 5c). In addition, these reporters provided signal on par with the positive control antibody–polymeric chelators when treated at similar concentrations (see SI Figure 11). Continuing studies are focused on providing substantial signal increases through encapsulation of lanthanide nanoparticles inside targeted MS2 capsids[46] to

DOI: 10.1021/acs.bioconjchem.5b00226
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detect single binding events within individual cells in complex cell populations.

CONCLUSIONS

The work presented describes a rapid and facile strategy for the attachment of full-length antibodies to the exterior surfaces of genome-free MS2 viral capsids. This method of attachment does not require a large excess of the antibody targeting moiety, mitigating some of the costs associated with obtaining targeted agents through other methods. The conjugates maintain the targeting affinity and specificity of the parent antibodies and have physical properties similar to those of the unmodified capsids. The hollow protein shells provide the advantage of various cargo (dyes, chelators, drug molecules) being attached to the interior surface without affecting the overall binding properties of the construct. MS2-mAb have been used to detect targets of interest via different methodologies, such as confocal microscopy, flow cytometry, and mass cytometry. While such constructs may be immunogenic, the labeling approach utilized herein provides the opportunity to utilize PEG molecules or "self-peptides"[47] to help mitigate these effects, and work in these areas is currently underway in our laboratories. Through extensive optimization of the reaction conditions and characterization of the corresponding conjugates, these studies have laid the foundation for the use of MS2-mAb as targeted in vivo imaging agents and drug delivery systems.

ASSOCIATED CONTENT

Supporting Information

Full experimental details and figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00226.

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Author Contributions

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

These studies were funded by the DOD Breast Cancer Research Program (Grants BC061995, BC100159, and W81XWH-14-0400 to M.B.F., M.E.F., and A.M.E., respectively) and the W.M. Keck Foundation. C.N. was supported by a Howard Hughes Medical Institute International Student Research Fellowship. I.L.A. was supported by the Genentech Fellowship through the U.C. Berkeley Chemical Biology program. G.P.N. was supported by grants from the NIH (U19 AI057229, U54CA149145, N01−HV-00242, 1U19AI100627, 5R01AI07372405, R01CA184968, 1 R33 CA183654, R33 CA183692), NIH-Baylor Research Institute (41000411217), NIH-Northrop Grumman Corp. (7500108142), CIRM (DR1-01477), DOD (OC110674, 11491122), the European Commission (Health.2010.1.2-1), FDA (HHSF223201210194C), and the Bill and Melinda Gates Foundation (OPP 1017093). LC-MS instrumentation was acquired with National Institutes of Health Grant 1S10RR022393-01. The authors thank Stacy Capehart for acquisition of the TEM images in this study.

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DOI: 10.1021/acs.bioconjchem.5b00226
Bioconjugate Chem. 2015, 26, 1590−1596

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Development of Oxidative Coupling Strategies for Site-Selective Protein Modification

Published as part of the Accounts of Chemical Research special issue "Synthesis, Design, and Molecular Function".

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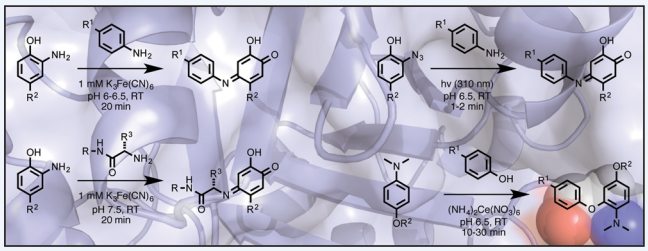
CONSPECTUS: As the need to prepare ever more complex but well-defined materials has increased, a similar need for reliable synthetic strategies to access them has arisen. Accordingly, recent years have seen a steep increase in the development of reactions that can proceed under mild conditions, in aqueous environments, and with low concentrations of reactants. To enable the preparation of well-defined biomolecular materials with novel functional properties, our laboratory has a continuing interest in developing new bioconjugation reactions. A particular area of focus has been the development of oxidative reactions to perform rapid site- and chemoselective couplings of electron rich aromatic species with both unnatural and canonical amino acid residues.



This Account details the evolution of oxidative coupling reactions in our laboratory, from initial concepts to highly efficient reactions, focusing on the practical aspects of performing and developing reactions of this type. We begin by discussing our rationale for choosing an oxidative coupling approach to bioconjugation, highlighting many of the benefits that such strategies provide. In addition, we discuss the general workflow we have adopted to discover protein modification reactions directly in aqueous media with biologically relevant substrates.

We then review our early explorations of periodate-mediated oxidative couplings between primary anilines and *p*-phenylenediamine substrates, highlighting the most important lessons that were garnered from these studies. Key mechanistic insights allowed us to develop second-generation reactions between anilines and anisidine derivatives. In addition, we summarize the methods we have used for the introduction of aniline groups onto protein substrates for modification.

The development of an efficient and chemoselective coupling of anisidine derivatives with tyrosine residues in the presence of ceric ammonium nitrate is next described. Here, our logic and workflow are used to highlight the challenges and opportunities associated with the optimization of site-selective chemistries that target native amino acids.

We close by discussing the most recent reports from our laboratory that have capitalized on the unique reactivity of *o*-iminoquinone derivatives. We discuss the various oxidants and conditions that can be used to generate these reactive intermediates from appropriate precursors, as well as the product distributions that result. We also describe our work to determine the nature of iminoquinone reactivity with proteins and peptides bearing free N-terminal amino groups.

Through this discussion, we hope to facilitate the use of oxidative approaches to protein bioconjugation, as well as inspire the discovery of new reactions for the site-selective modification of biomolecular targets.

INTRODUCTION

The development of new methods for covalent bond formation that takes place rapidly, under mild conditions, and with high levels of chemoselectivity has transformed the ways in which chemists approach the synthesis of new materials with advanced functional properties. One very promising area capitalizes on reactions that proceed in water in the presence of polar functional groups, enabling the generation of multicomponent structures that comprise both biological and abiotic components. Many of these hybrid materials have provided new avenues for drug delivery, molecular sensing, catalysis, and controlled material growth, representing a compelling new frontier for the design and generation of molecular complexity.

To maintain the folded structure of most biomolecules, modification strategies must take place in buffered aqueous solution at near-neutral pH and temperatures below 37 °C. Very few reactions in the canon of organic chemistry meet these requirements, and fewer still possess sufficiently high rate constants to proceed readily at the micromolar concentrations of most biomolecular substrates. To fill this void, recent years have seen much research activity toward developing chemical strategies that complement and extend the tried-and-true methods of the field.[1−4] Many of these reactions fall under the moniker of "click chemistry", originally defined by Sharpless

Received: March 20, 2015
Published: June 9, 2015

DOI: 10.1021/acs.accounts.5b00139
Acc. Chem. Res. 2015, 48, 1971−1978

and co-workers as reactions that are modular, high yielding, stereospecific, and free of byproducts.[5] In addition to these small molecule-based strategies, a growing number of powerful enzymatic labeling methods have been developed to achieve protein bioconjugation with high degrees of sequence specificity.[6] Together, these new synthetic tools have had an enabling impact on the field of Chemical Biology, and they are rapidly effacing the divisions between organic synthesis and biosynthesis.

Over the past decade, our laboratory has invested considerable effort developing new oxidative coupling strategies for protein bioconjugation. In their most general form, these reactions involve the addition of electron-rich aromatic species to electrophiles generated *in situ*. Both carbon−heteroatom and carbon−carbon bonds can be formed in this fashion, and the products are stable toward pH extremes, heating, nucleophiles, oxidants, and reductants. Through reaction screening, highly specific coupling pairs have been developed, and most of these reactions proceed rapidly at high dilution. This feature allows whole biomolecules to be joined efficiently despite substantial steric bulk and the presence of hundreds of polar spectator groups. In a previous Account, we detailed the use of these strategies for the synthesis of targeted delivery materials for *in vivo* applications.[7] Here, we instead focus on the ways in which these strategies were developed, with the goal of providing both insight into this new reaction class and a blueprint for how protein bioconjugation reactions can be developed.

From the onset, we recognized a number of advantages to an oxidative manifold. First, the majority of biomolecules can tolerate a reasonable measure of oxidative conditions, as we have confirmed many times during our studies. The functional groups of most concern include free cysteines, methionines, tryptophans, and vicinyl diols on carbohydrate groups. Indeed, during the course of our studies, we have observed unwanted oxidations under some conditions that we screened. Fortunately, there exists a wealth of chemical reagents with variable redox potentials and preferences for mechanistically distinct pathways (e.g., two electron vs one electron), suggesting that many of these undesired side reactions could be avoided through judicious oxidant and substrate choices. Second, the large number of water-soluble oxidants in the form of salts facilitates reaction screening. In addition, because most oxidants have pH-variable redox potentials, buffer pH can be used to tune reactivity. Third, using buffered solutions facilitates the stabilization of charged or highly polarized intermediates that result from oxidative conditions. Fourth, in most cases, substrates prior to oxidation are nucleophilic while their oxidized counterparts are electrophilic. This, in effect, allows the unoxidized starting material to behave as a masked or "protected" functional group that can be unveiled by oxidation. This trait is particularly useful because the majority of intrinsically reactive species on proteins and nucleic acids behave as nucleophiles that can be used to intercept these oxidized species. Finally, most oxidants are easy to handle, requiring little if any precaution in excluding air or water.

Throughout the course of these studies, we have focused on developing new reactions directly in buffered aqueous media at low concentrations on biologically relevant substrates. Although such an approach can present its own set of challenges, such as structural elucidation of the resultant products, chemoselectivity issues can be explored upfront and the reoptimization of reactions for aqueous conditions is unnecessary. Furthermore, as will be described below, the unique properties of water as a solvent[5] allow for distinct modes of reactivity that either are not replicable in organic solvents or are highly inefficient. While knowledge of the product structures is crucially important, structural confirmation studies are often among the last steps in our workflow. Instead, our early screening studies rely heavily on either matrix-assisted laser desorption ionization time of flight (MALDI-TOF) or liquid chromatography/mass spectrometry (LC/MS) studies, most often in the context of small peptides bearing the functional group of interest using natural or unnatural amino acids. Adducts are then subjected to tandem mass spectrometry to determine the residue(s) involved in the observed reactivity. In many cases, these studies, when combined with mechanistic considerations, give hints to possible product structures. When high levels of chemoselectivity have been achieved or when structural information may facilitate improving reaction efficiencies, small molecule model systems are prepared for full structural elucidation studies, typically by combinations of 1D and 2D NMR characterization techniques.

DEVELOPMENT OF A NEW OXIDATIVE BIOCONJUGATION STRATEGY

The central design lead for these strategies was provided by a new coupling reaction reported by our laboratory in 2006.[8] We observed that in the presence of sodium periodate, *N*-acyl phenylenediamines (such as **1**, Figure 1a) underwent a clean trimerization reaction to afford red dye derivatives (**3**) described by Bandrowski in the late 1890s.[9] This reaction proceeded in minutes in aqueous solution buffered at pH 6.5 and was unaffected by the presence of complex biomolecules and polar functional groups. From this starting point, we first

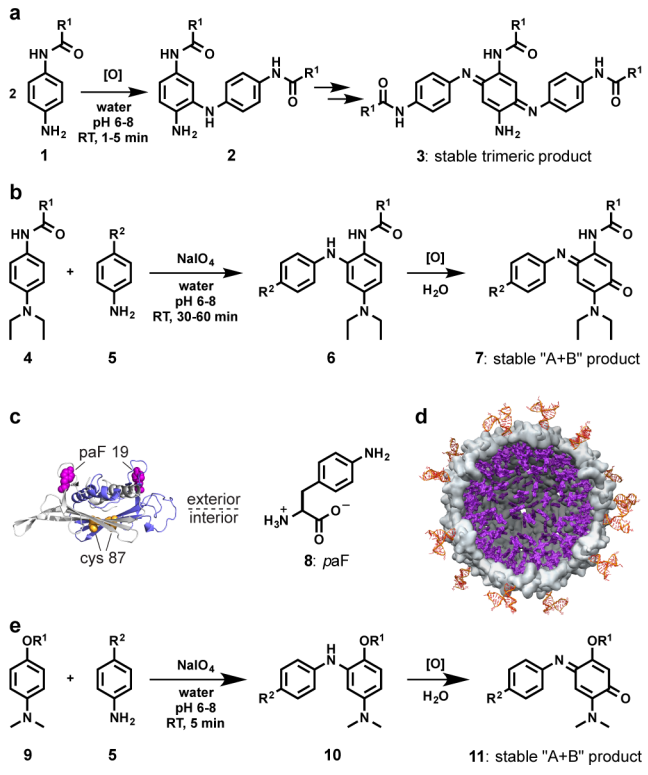


Figure 1. Early examples of oxidative coupling reactions involving (a,b) *N*,*N*-dialkyl phenylenediamines and (e) anisidines. Anilines on MS2 protein dimers (*p*aF residues) are indicated in part c. In part d, DNA aptamers appear in orange and porphyrins appear in purple.

DOI: 10.1021/acs.accounts.5b00139
Acc. Chem. Res. 2015, 48, 1971−1978

sought to stop the trimerization reaction through the addition of alkyl groups to the aniline nitrogen, Figure 1b. The resulting *N*,*N*-dialkyl phenylenediamine **4** was unable to form the trimer, as expected; however, this species retained its ability to react with primary anilines, leading to the efficient formation of a stable "A + B" product. Steric hindrance from the *N*,*N*-dialkyl substitution presumably prevented addition of a second aniline at the *ortho* position. Similar to the trimeric dye derivatives, these compounds exhibited high levels of stability, with no degradation observed over 12 h from pH 2−11.

Initial screening studies quickly determined that derivatives of general structure **4** couple rapidly to aniline groups on proteins (**8**) in the presence of periodate with little-to-no participation by native functional groups, Figure 1c,d. The most impressive feature of this strategy is its compatibility with a broad range of molecules, including proteins, nucleic acids, polymers, and small molecule chromophores. The optimized conditions use pH 6−6.5 phosphate buffer with 1 mM periodate. Coupling times range from 10 min for small molecules to 1 h for macromolecules. Following the reaction, periodate is quenched with tris(2-carboxyethyl)phosphine (TCEP) or removed by gel filtration through a Sephadex column.

To generate protein substrates for these reactions, we have shown that aniline groups can be introduced directly using amber codon suppression[10] or through native chemical ligation using an aniline-containing cysteine derivative.[8] Alternatively, ketones introduced at the N-termini of proteins through transamination[11,12] can be coupled to aniline-containing alkoxyamines through oxime formation.[13] Finally, labeling lysine residues with isatoic anhydride provides ready access to aniline derivatives.[8] However, these substrates show diminished reactivity since they are vinylogous amides and suffer the usual lack of site selectivity of lysine modification reactions.

Subsequent studies showed that the functional group tolerance and high efficiency of this reaction under dilute conditions were useful to prepare complex bioconjugates for targeted delivery applications.[10,14,15] This coupling chemistry was used to attach DNA aptamers[16] to the external surfaces of MS2 capsids, providing a means to target tyrosine kinase 7 proteins overexpressed on leukemia cells. Elaboration with porphyrin groups inside of the capsids allowed prodigious amounts of singlet oxygen to be produced upon photoillumination, resulting in efficient killing of only the targeted cells.[15] In the broader sense, these initial investigations demonstrated the potential of oxidative coupling reactions to prepare biomolecular materials of high complexity.

ANISIDINES AS ANILINE COUPLING AGENTS

While reactions involving *p*-phenylenediamine substrates provided a reliable method to label aniline-containing proteins, extended reaction times (up to 2 h) in the presence of periodate resulted in competitive background oxidation of native sulfur-containing residues. Mechanistic considerations of two aspects of this reaction pointed to a rate-determining step associated with oxidation of the phenylenediamine substrate. First, periodate treatment of **4** alone (i.e., in the absence of aniline) led to little observable oxidation of the substrate within the first 5 min. Second, the clean trimerization of **1** suggested that oxidation must be rate determining; otherwise, a low concentration of the nucleophilic species would be expected in solution, and the trimerization would be greatly mitigated. We anticipated that replacement of the *N*-acyl substituent with a more electron-donating moiety would facilitate the oxidation event and increase the overall rate of the reaction. In practice, we found that *N*,*N*-dialkyl-*p*-anisidine derivatives (**9**) undergo rapid oxidative coupling (2−5 min) with aniline derivatives when treated with periodate, Figure 1e.[17] This reaction was used to integrate fluorescein donors and porphyrin acceptors on the interior and exterior surfaces of a viral capsid to achieve photocatalysis at broad wavelengths.[17] To date, this reaction remains among the fastest techniques developed within our laboratory, and the stability of the anisidine coupling partner renders this reaction an excellent choice for many applications.

OXIDATIVE COUPLINGS WITH ELECTRON-RICH NATURAL AMINO ACIDS

The use of *p*-phenylenediamine and anisidine substrates in oxidative couplings with aniline derivatives served as a starting point for another distinct set of bioconjugation reactions. In particular, we envisioned single electron oxidation of these substrates (general structure **4** or **9**) to generate a highly electrophilic radical cation that would be subject to reaction with either a tyrosine or a tryptophan residue. In the former case, beautiful work from the Kodadek laboratory had demonstrated that the use of $Ru(bpy)_3^{2+}$ under visible light illumination effectively generates tyrosyl radicals capable of cross-linking proteins through an oxidative dimerization reaction.[18] Here, we recognized that the key to the success of our proposed strategy would be identification of an appropriate oxidant and substrate to differentiate between the tryptophan and tyrosine residues while minimizing undesired reactions with other side chains.

We began by exploring oxidative conditions to effect the coupling of **4** or **9** with peptide substrates under aqueous, unbuffered conditions.[19] Angiotensin II (a tyrosine-containing peptide) and melittin (a tryptophan-containing peptide) were screened in parallel to differentiate the reactivity of the two most electron-rich aromatic amino acids. Initial screens focused on the identification of an oxidant capable of delivering a single, stable adduct of **4** with the peptides. Common two-electron oxidants proved incapable of controllably delivering coupled products, with either no reaction or overaddition of the substrate observed. A screen of one-electron oxidants, however, identified ceric ammonium nitrate (CAN) as a promising oxidant for the desired transformation, having controllably coupled **4** to both angiotensin and melittin. Subsequent MS/MS experiments clearly demonstrated modification only at the electron-rich residues. Further buffer and pH screens revealed that 50 mM, pH 6.0 bis(TRIS) proved optimal to maintain reactivity while effectively buffering the solution (10 mM buffer solutions do not effectively buffer 1.5 mM solutions of CAN). Although phosphate buffers cause precipitation of CAN from aqueous solutions, this feature provides a convenient method to remove cerium salts upon completion of the reaction.

N,*N*-Dialkylated anilines bearing an additional electron donating group in the *para* position were the most reactive, with **4** and **9** proving optimal. Less electron-rich substrates were unreactive, and primary anilines exhibited overmodification. Importantly, studies with compound **9** demonstrated efficient reactivity with angiotensin but no reactivity with melittin (Figure 2b−d), and MS/MS analysis of this coupling confirmed modification only at the tyrosine residue. Together, these data pointed to a chemoselective coupling of anisidine derivatives with tyrosines. At this point, small molecule model studies were

DOI: 10.1021/acs.accounts.5b00139
Acc. Chem. Res. 2015, 48, 1971−1978

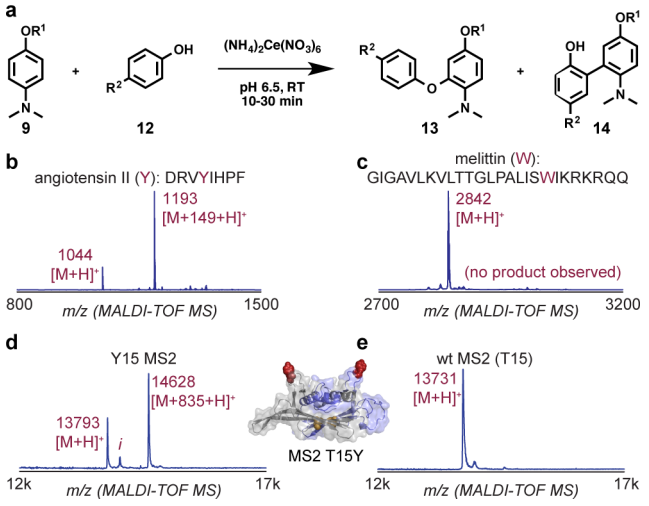


Figure 2. Addition of anisidines to tyrosine residues in the presence of CAN. (a) Two isomeric products are obtained. Reaction selectivity was confirmed on (b,c) peptides and (d,e) MS2 capsids bearing or lacking a tyrosine in position 15 (red).

undertaken to determine the structure of the conjugates, as summarized in Figure 2a.

The above reactions constitute an effective means of performing oxidative couplings of electron-rich canonical amino acids with aniline derivatives.[19] A few features of these reactions are worthy of mention. First, the use of CAN as oxidant allows one of the key limitations of oxidative couplings utilizing periodate to be circumvented: the oxidative degradation of 1,2-diols present in sugars on glycoproteins. While CAN is a powerful oxidant, subsequent studies on many proteins have indicated that it is quite compatible with a variety of substrates if extended periods of incubation with high concentrations are avoided (3:1 with respect to the ansidine coupling partner greatly mitigates any unwanted side oxidations). The use of bis(TRIS) buffer appears to be unique in maintaining reactivity while buffering the solutions. The basis of this phenomenon remains unclear; however, a notable color change takes place upon addition of solutions of CAN to bis(TRIS), potentially signifying the formation of a complex responsible for the subsequent chemistry.

OXIDATIVE COUPLINGS OF AMINOPHENOLS AND CATECHOLS

In our earliest work, we explored the coupling of phenylenediamines (**1**) with *o*-aminophenols (**15**, Figure 3a) generated on viral capsids through tyrosine modification.[20] Determination of the product structure proved challenging; the clean reactivity observed on proteins could never be translated to small molecule analogues. Returning to aminophenol substrates several years later, we recognized that a pitfall of the earlier studies was that both coupling partners can be subject to oxidation. Thus, we hoped that utilizing toluidine-derived coupling partners would greatly simplify the reaction with putative *ortho*-iminoquinone intermediates. Here, we believed this reaction would benefit from the higher degree of polarization in the alkene relative to a *para*-iminoquinone, promoting a faster conjugate addition than any reactions we had previously explored.

At the outset of these studies, however, we were cognizant that in our proposed coupling, a rate limiting oxidation could

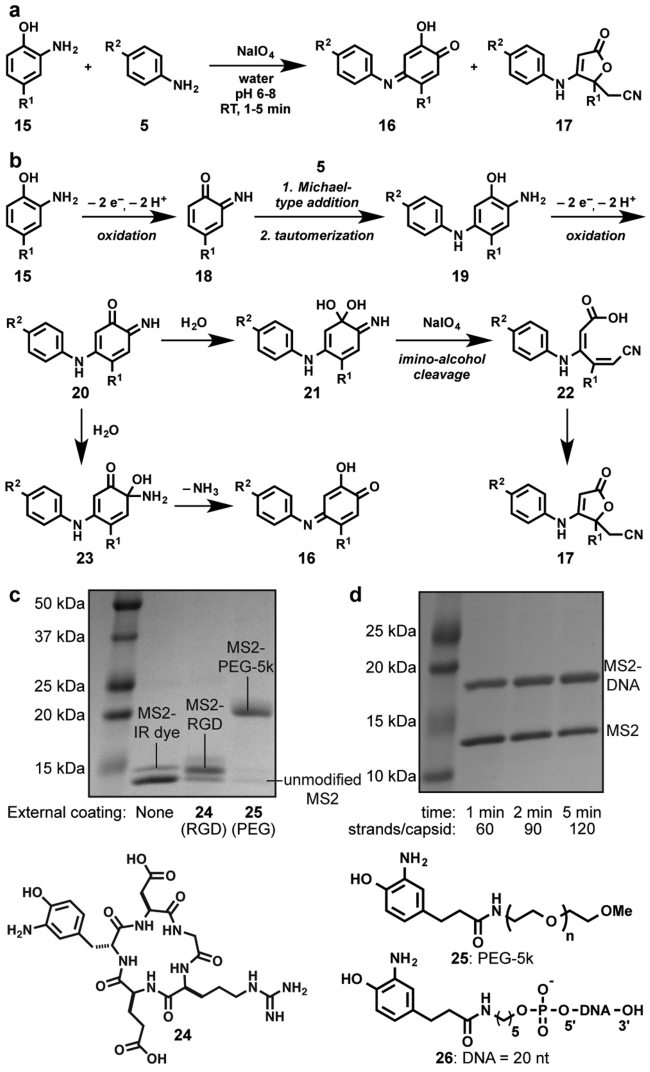


Figure 3. Oxidative couplings with aminophenols. The overall reaction is shown in part a, and proposed mechanisms are provided in part b. The modification of *p*aF MS2 capsids internally labeled with an IR dye appears in part c, and the attachment of DNA to *p*aF MS2 capsids is shown in part d.

complicate this bioconjugation reaction since unoxidized aminophenol would be present in solution and could potentially participate in unwanted reactions. Studies describing the oxidation of *o*-aminophenol substrates with various substitution patterns demonstrated that facile dimerization of such substrates can take place. Surprisingly, relatively subtle structural alterations resulted in dramatically different outcomes during the reactions.[21]

Despite these reservations, we elected to examine this reaction in buffered aqueous conditions under periodate-mediated oxidation. A pH 6.5 phosphate-buffered solution of 2-amino-*p*-cresol and toluidine (1:1) was treated with periodate for 5 min. HPLC analysis of the crude reaction mixture showed an uneven mixture of two products and no starting materials. Purification provided sufficient material for structural characterization of the major product through X-ray crystallographic analysis, which revealed that an unprecedented oxidative ring contraction of the aminophenol-containing ring had occurred, giving rise to compound **17** (Figure 3a).[22] A reasonable

DOI: 10.1021/acs.accounts.5b00139
Acc. Chem. Res. 2015, 48, 1971−1978

mechanistic hypothesis for its formation is provided in Figure 3b.

The minor product could not be obtained in sufficient quantity for structural determination. The high-resolution mass spectrum of this compound suggested that only one nitrogen atom was present in the structure, and thus, we anticipated that use of the corresponding catechol in the coupling reaction would provide an alternative route to the minor product through similar reactivity.[23,24] Indeed, a single product that coeluted by HPLC with the minor product above resulted from oxidative coupling with toluidine. This aniline/catechol coupling is highly efficient and provided material for structural characterization. NMR analysis revealed the product to be that expected from direct conjugate addition/oxidation of the *o*-quinone species, **16**.

A time screen in the context of protein-based substrates revealed that the reaction often reached completion within 30 s. This exceptionally high rate of reaction suggested that although periodate was used as an oxidant, side reactions with other oxidation-sensitive functional groups could likely be mitigated. As a demonstration of the compatibility of this reaction with sugars, in couplings of aminophenol-labeled DNA to aniline-labeled antibody Fc domains,[13] oxidation of the glycans could be completely suppressed by the addition of 10 mM mannose (10× with respect to periodate). As a testament to the versatility of this protocol, a number of hybrid biomolecular materials have been prepared using this reaction.[13,25−27] Additional examples of MS2 capsid modification appear in Figure 3c,d.

Here, a practical note concerning the introduction of aminophenols onto coupling partners of interest is worth mention. The catechol and aminophenol substrates are not stable to prolonged storage in solution. Instead, we prefer to use an *o*-nitrophenol moiety that can be readily reduced with sodium dithionite ($Na_2S_2O_4$) prior to the desired coupling. The nitrophenol congener is very stable and can be stored indefinitely in solution. In addition, we prefer the aminophenol to the catechol due to the ability to store it as the nitrophenol congener, despite the fact that the catechol derivative gives a single product.

BEYOND PERIODATE: FERRICYANIDE-MEDIATED OXIDATIVE COUPLINGS

Although the periodate-mediated coupling of aminophenols with aniline derivatives is an extremely reliable reaction, two products are produced, and some unwanted oxidation was observed in a few scenarios (albeit surmountable by judicious choice of equivalents or through the use of appropriate additives). This prompted us to screen other oxidants for this coupling, which revealed a few other reagents that were capable of initiating the desired coupling to varying degrees. These were CAN, Ag(I) salts, Cu(II) salts, and potassium ferricyanide [$K_3Fe(CN)_6$]. Of these, we pursued ferricyanide since it is a mild oxidant and reactions employing this reagent gave excellent levels of conversion.[28] An additional attractive feature was that, unlike CAN and Ag(I) salts, buffer incompatibilities were not of concern. Importantly, we found this oxidant maintains the highest levels of compatibility with oxidation-sensitive functional groups; proteins containing free thiols, sugars, methionine residues, and tryptophan residues are not oxidized by $K_3Fe(CN)_6$, even in vast excesses of reagent over long periods of time.

Further exploration of this coupling with toluidine revealed that a single product (**16**, Figure 4a) is formed, likely because

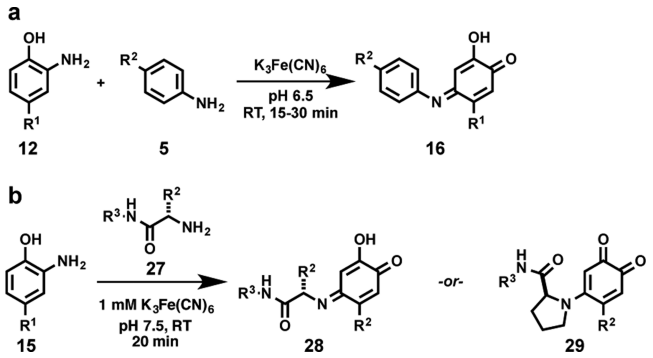


Figure 4. Modification of (a) anilines and (b) N-terminal residues using aminophenols and ferricyanide.

ferricyanide cannot perform a concerted two-electron oxidation necessary to generate the ring-contracted product. These products are exceedingly stable over a broad rage of pH values (2−10) and temperatures (50 °C for 18 h showed no loss in product). Additionally, large excesses (500 equiv) of many nucleophilic and reducing species are generally compatible with the product at room temperature over a period of 18 h, with the only major exception being the use of anisidine wherein a ∼75% reduction in product was observed.[28]

While this reaction is slower than the periodate-mediated coupling (typically 15−30 min versus 2−5 min), comparable levels of conversion are generally achieved. A pH screen using MS2 viral capsids bearing aniline side chains showed 6.0−6.5 to be optimal. Interestingly, at higher pH (7.0−7.5), we observed small amounts of secondary modifications (higher pH values had not been assessed with the periodate-mediated coupling). At this time, the precise site of these extra modifications was unknown (see the section below), though we were able to suppress such unwanted reactivity effectively through the addition of 1−10 mM imidazole.[24,28]

N-TERMINAL CHEMISTRY AND APPLICATIONS

As mentioned above, coupling of *o*-quinone/iminoquinone intermediates with aniline derivatives was found to be highly selective under slightly acidic conditions; however, at higher pH, further adducts were observed. Literature precedents from Messersmith,[23] Kodadek,[24] and others[29−32] describing the reaction of native amino acids and proteins with putative *o*-quinone precursors are well documented in the literature. Here, we hoped to both identify the source of this reactivity and develop conditions to achieve an efficient and selective reaction.

To determine the nature of the observed reactivity and optimize for selectivity,[24] we again chose to use angiotensin and melittin as model peptides that contain many of the common reactive functional groups found on proteins, including free N-terminal amino groups, lysine, arginine, histidine, tryptophan, and tyrosine residues. These peptides were treated with 2-amino-*p*-cresol and ferricyanide for 20 min under varying pH (5.5−8.5), and the reactions were analyzed by MALDI-TOF.[33] As previously observed, higher levels of reactivity were observed on both peptides with increasing pH, and nearly quantitative conversion was achieved on angiotensin at or above pH 7.5. Importantly, in each case, only a single modification was observed. Tandem MS/MS experiments on

DOI: 10.1021/acs.accounts.5b00139
Acc. Chem. Res. 2015, 48, 1971−1978

the crude coupling with angiotensin clearly demonstrated that the N-terminal amino acid was involved in the reaction. Given the pH dependence of the reaction, we deduced that the N-terminal amino group was responsible for the reaction, giving rise to a product of analogous structure to that from coupling with toluidine derivatives (Figure 4b). This hypothesis was bolstered by the emergence of the characteristic purple/red color of the product during the course of the reaction. Furthermore, an N-terminal pyroglutamate peptide was completely unreactive, confirming the necessity of a free N-terminal amino group. Next, we probed the effect of the N-terminal residue on the reaction efficiency by screening a series of X-ADSWAG peptides (X = canonical amino acids except cysteine, due to known cross reactivity). While all N-termini were reactive (average yield of 65% by MALDI-TOF analysis), the N-terminal proline peptide consistently showed the highest levels of reactivity in terms of both rate and conversion. In transitioning to protein-based substrates, the necessity of an N-terminal proline was more stringent in order to achieve high levels of conversion. However, useful levels have been achieved with other amino acids.[33]

Interestingly, we found the coupling efficiency of these reactions could not be improved through the use of more equivalents of the aminophenol coupling partner. In fact, the use of excess aminophenol during the coupling resulted in notably worse conversion. We attribute this result to the fact that at higher equivalents of **15**, the rate-limiting oxidation allows the unoxidized aniline-containing starting material to undergo competitive homocoupling, which has been observed in reactions using either high numbers of equivalents of the aminophenol or no coupling partner at all. Mechanistic studies of this reaction were simplified by performing couplings with the catechol derivative where dimerization was not observed. Kinetic studies of the N-terminal proline coupling with 4-methylcatechol by monitoring the appearance of the distinct UV−vis absorption at 520 nm allowed a second order rate constant of 44 ± 4 M^{-1} s^{-1} to be determined when using a vast excess of oxidant, which allowed us to overcome the rate determining oxidation. The corresponding reaction with aniline derivatives was too fast to allow an accurate determination of the second order rate constant with this method.[33]

PHOTOCHEMICAL COUPLINGS AND THEIR APPLICATIONS

When aminophenols are used, both the periodate and the ferricyanide coupling reactions presumably involve the formation of a highly reactive iminoquinone intermediate. We have recently discovered an interesting way to access this intermediate photochemically, thus avoiding the use of any additional oxidants.[34] This method involves the irradiation of *o*-azidophenols, which lose nitrogen to afford a nitrene species that immediately undergoes redox tautomerization with the neighboring phenol (Figure 5a).[35−37] These intermediates couple rapidly to aniline groups as described above, yielding the same products after additional aerobic oxidation. This reaction can be quite efficient for the modification of anilines on proteins, requiring as little as 2 min of irradiation at 310 nm to achieve nearly complete conversion for small molecules and >60% yield after 5 min of irradiation for PEG molecules. The *o*-azidophenols can be stored for extended periods at 0 °C in the absence of light.

Because irradiation is required to initiate coupling, this method offers particular promise for generating patterned

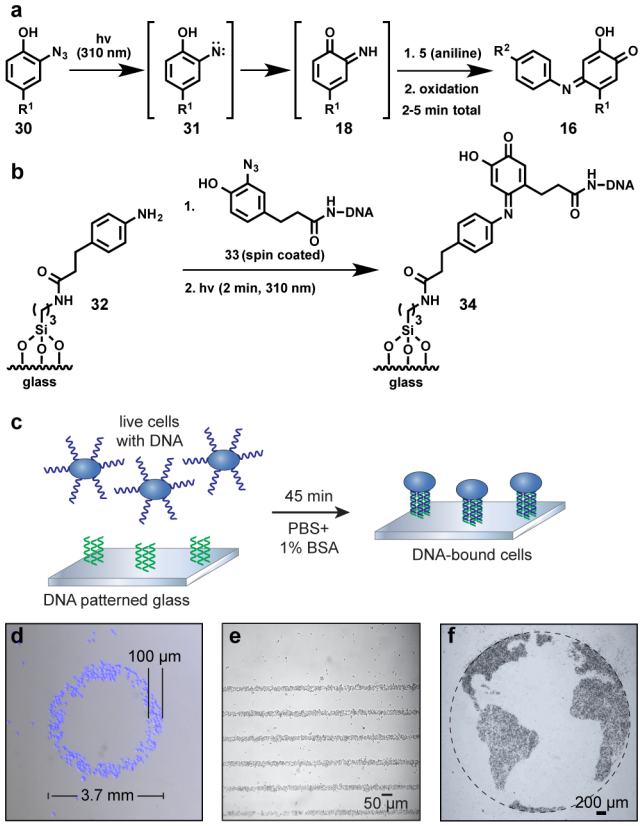


Figure 5. Photochemical coupling reactions based on azidophenols. The basic reaction is shown in part a and its application to DNA surface patterning is shown in part b. (c) Live cells bearing complementary DNA strands can be captured by the surface patterns, as shown for (d) vascular smooth muscle cells and (e,f) yeast.

arrays of biomolecules on surfaces. The use of traditional photolithography for this purpose is hampered by the fact that most photoresists react chemically with biomolecules, rendering them incompatible. Via the azidophenol coupling reaction, a "resist-free" version of photopatterning has been achieved on aniline-coated glass substrates prepared using silane chemistry.[34] A solution of DNA strands bearing azidophenol groups at their 5′-termini is then applied via spin coating. Irradiation through an appropriate photomask results in the conjugation of the DNA molecules to the surface in the exposed regions (Figure 5b). The unbound DNA can be removed from the surface through simple rinsing to leave the desired pattern behind.

One application of this patterning technique is the capture of living cells through DNA hybridization, Figure 5c. We have previously published methods for the attachment of synthetic DNA strands to both mammalian[38,39] and nonmammalian[40] cells, giving them the ability to adhere to surfaces bearing the sequence complements. This methodology is ideal for placing cells in controlled environments for use in diagnostic assays and synthetic biology applications. The results of this process using photopatterned DNA are shown in Figure 5d−f.[34] We are currently exploring applications of this method for the generation of well-defined arrays containing multiple cell types, with the goal of studying the ways in which neighboring cell interactions govern cancer cell morphology, migration, and drug response. In addition, we are using this method to

DOI: 10.1021/acs.accounts.5b00139
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combine different types of microorganisms to study metabolite sharing and other symbiotic behaviors.

CONCLUSIONS

Through these studies, a new set of bioconjugation reactions has been developed based on oxidative coupling pathways.

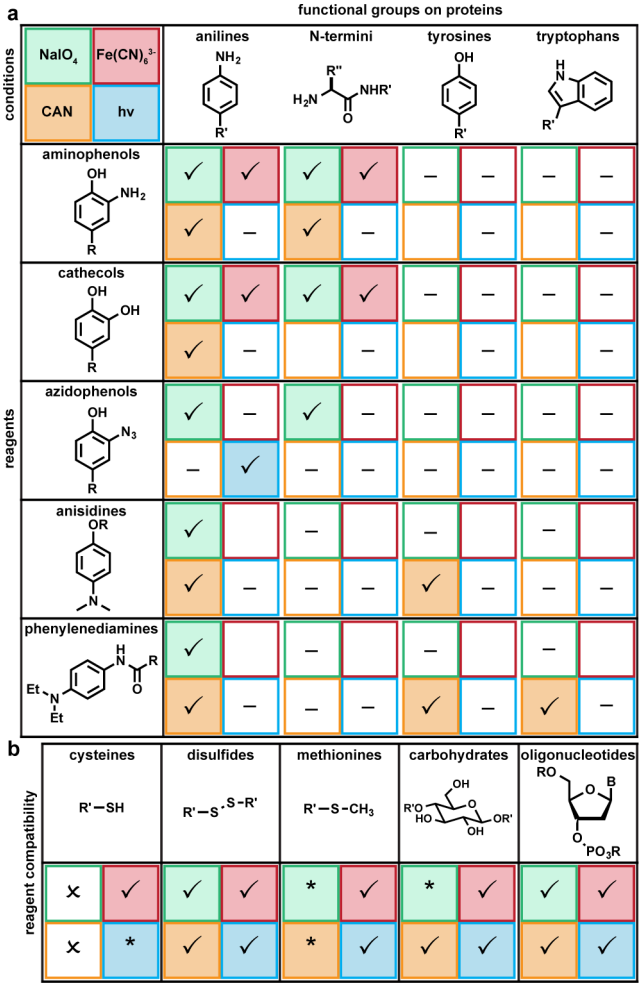

Figure 6. Chemoselectivity and functional group compatibility of the new oxidative protein modification reactions. (a) Successful combinations of reagents, protein functional groups, and oxidants are indicated by the check marks. The dashes indicate combinations known not to react, and empty boxes indicate that the combination has not yet been evaluated. (b) The compatibilities of the four basic sets of reaction conditions are indicated for oxidation-sensitive protein functional groups. An ∗ indicates that side reactions can be suppressed through the use of additives.

Many of these reactions are the first of their kind, providing both useful methods for synthetic group attachment and design leads for future strategies. Taken as a whole, these methods offer a particularly attractive set of features. First, they exhibit very high coupling rates, with typical reactions occurring in 2−20 min even at micromolar concentrations. This capability avoids prolonged exposure to the reaction conditions, minimizes opportunities for biomolecule denaturation, and is highly advantageous for time-critical applications, such as radiolabeling. In addition, the rapid rates of these reactions are only minimally influenced by steric factors, allowing the efficient coupling of full-sized biomolecules in similar amounts of time. Our studies have indicated that the products are highly stable with respect to hydrolysis, reducing agents, oxidants, and strong nucleophiles. The oxidative strategies described herein also rely on easily prepared, inherently water-soluble functional groups and use oxidants that can be easily removed from the product mixtures. Most significantly, by subtly changing the reaction conditions, the reactant structures, or both, these methods can be used to target a variety of natural and unnatural functional groups with high selectivity, offering a greater range of substrates for which they can be used, as summarized in Figure 6. Considering the broad range of electron-rich aromatic and heterocyclic molecules that has yet to be explored in this chemistry, it is likely that many additional transformations can be discovered through the application of the reaction development workflow that has been established. The development of new oxidative coupling strategies and the synthesis of complex biomolecular materials through their use are proceeding apace in our laboratory.

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Notes

The authors declare no competing financial interest.

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Matthew B. Francis received his undergraduate degree in Chemistry from Miami University in Oxford, OH, in 1994. From 1994 to 1999, he attended graduate school at Harvard University, working with Prof. Eric Jacobsen. He then moved to UC Berkeley, where he was a Postdoctoral Fellow working with Prof. Jean Fréchet. Matt started his independent career in the UC Berkeley Chemistry Department in 2001, and he is also a faculty member at the Lawrence Berkeley National Laboratory.

ACKNOWLEDGMENTS

The development of these reactive strategies was generously supported by the NIH (Grant R01 GM072700) and the DOD Breast Cancer Research Program (Grant BC061995). A.M.E. was supported by a DOD BCRP postdoctoral fellowship (Grant W81XWH-14-0400). The authors thank Dr. Allie Obermeyer and Dr. Kristen Seim for very helpful discussions.

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